

Localization of metabotropic glutamate receptors in the outer plexiform layer of the goldfish retina

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Abstract We studied the localization of metabotropic glutamate receptors (mGluRs) in the goldfish outer plexiform layer by light- and electron-microscopical immunohistochemistry. The mGluR1 α antibody labeled putative ON-type bipolar cell dendrites and horizontal cell processes in both rod spherules and cone triads. Immunolabeling for mGluR2/3 was absent in the rod synaptic complex but was found at horizontal cell dendrites directly opposing the cone synaptic ribbon. The mGluR5 antibody labeled Müller cell processes wrapping rod terminals and horizontal cell somata. The mGluR7 antibody labeled mainly horizontal cell dendrites invaginating rods and cones and some putative bipolar cell dendrites in the cone synaptic complex. The finding of abundant expression of various mGluRs in bipolar and horizontal cell dendrites suggests multiple sites of glutamatergic modulation in the outer retina.

Keywords Metabotropic glutamate receptor · Retina · Electron microscopy · Immunohistochemistry · Bipolar cell · Horizontal cell · Goldfish, *Carassius auratus* (Teleostei)

Introduction

Stimulation of vertebrate photoreceptors by light decreases the release of glutamate (Cervetto and MacNichol 1972;

Murakami et al. 1972; Kaneko and Shimazaki 1976), which is sensed by glutamate receptors (GluRs) in the dendrites of second-order neurons. Glutamate receptors are roughly divided into ionotropic and metabotropic receptors (Eccles and McGeer 1979). Ionotropic receptors (iGluRs) form an integral ion channel, whereas metabotropic receptors (mGluRs) mediate responses through indirect mechanisms involving signal transduction cascades.

So far, eight different mGluRs have been cloned and classified into three groups based on their sequence similarities, second-messenger cascade, and pharmacology: group I comprises mGluR1 (Houamed et al. 1991; Masu et al. 1991) and mGluR5 (Abe et al. 1992) and their splice variants (1a–d and 5a–b); group II contains mGluR2 and mGluR3 (Tanabe et al. 1992; Tanabe et al. 1993); and group III includes mGluR4 (Tanabe et al. 1993), mGluR6 (Nakajima et al. 1993), mGluR7 (Okamoto et al. 1994; Saugstad et al. 1994), and mGluR8 (Duvoisin et al. 1995), and their splice variants (4a–b, 7a–b, 6a–b, and 8a–b). The lack of specific pharmacological tools to distinguish between mGluRs within a group makes it difficult to link a particular receptor type to a certain response property (for reviews, see Thoreson and Ulphani 1995; Schoepp et al. 1999).

In the retina, mGluRs play an important role in the transmission and modulation of visual signals. Light responses of ON-type bipolar cells (ON BCs) of a number of species, for instance, are sensitive to substances/drugs that target group III mGluRs (Shiells et al. 1981; Slaughter and Miller 1981; Thoreson and Miller 1993). The receptor activated by these drugs is most likely mGluR6 (Nakajima et al. 1993; Akazawa et al. 1994; Masu et al. 1995; Ueda et al. 1997; Vardi and Morigiwa 1997; Laurie et al. 1997; Vardi et al. 2000). In fish, amphibians, and mammals, group III mGluRs modulate the glutamate release of cones (Koulen et al. 1999; Hirasawa

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et al. 2002; Hosoi et al. 2005). Furthermore, pharmacological activation of both group I and group III mGluRs modulates horizontal cell (HC) responses (Nawy et al. 1989; Takahashi and Copenhagen 1992; Linn and Gafka 1999), suggesting that multiple mGluRs must be present in the fish outer plexiform layer (OPL).

At the moment, however, data available on the identity and distribution of the various mGluRs in the teleost outer retina are scarce (Yazulla et al. 2001; Klooster et al. 2001). We have therefore performed a light and electron microscopy study of the mGluRs present in the goldfish OPL. An antibody against the ON BC marker, protein kinase C (PKC; Negishi et al. 1988; Suzuki and Kaneko 1990; Yazulla and Studholme 1992), has been used in order to identify possible localization in mixed-input ON BCs (ON MBCs).

Materials and methods

Subjects

Goldfish (*Carassius auratus*), 12–15 cm standard body length, were obtained from a commercial supplier and maintained at 16°C in aerated tanks filled with tap water circulating through a biological filter system. The fish were fed and kept on a 12 h/12 h light-dark cycle. All animal experiments were approved by the ethical committee of the Royal Netherlands Academy of Arts and Sciences, acting in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Preparation

Dissections took place early in the morning (between 7 a.m. and 9 a.m.) under ambient light. The fish used in this study were therefore all in the light phase of their circadian regime. After cervical transection, the cornea and lens of light-adapted animals were removed, and the remaining eyecups were cut in half through the equator. Half-eyecups were placed vitreous-side-down on a Millipore filter (13 mm diameter, 8 µm pore size; Millipore, Amsterdam, The Netherlands) placed on a filter holder. Suction was applied to remove the vitreous; the sclera and retinal pigment epithelium were peeled away.

Light microscopy

Retinas were fixed at room temperature for 10 min in 0.1 M phosphate-buffered 4% paraformaldehyde (pH 6.5) and for another 10 min in 0.1 M sodium carbonate-buffered 4% paraformaldehyde (pH 10.4). These short fixation times were chosen to prevent loss of antigenicity. After being rinsed in 0.1 M phosphate buffer (PB, pH 7.4), the tissue was

cryoprotected at room temperature for 30 min in PB containing 12.5% sucrose and for 1–2 h in PB containing 25% sucrose.

The pieces of retina, still attached to the filter, were embedded in Tissue Tek (Sakura Finetek Europe, Zouterwoude, The Netherlands) in an aluminum boat and frozen in liquid nitrogen. Sections (8–10 µm thick) were cut in a cryostat, mounted on poly-L-lysine-coated slides (Menzel-Gläser, Braunschweig, Germany), dried and stored in a non-frost-free freezer at –20°C until use.

Retinal sections were washed (2×, 10 min) in phosphate-buffered saline (PBS), blocked for 20 min in PBS with 2% normal goat serum (NGS; Jackson ImmunoResearch Lab, West Grove, USA), incubated for 18–48 h with the primary antibody (for dilutions, see Table 1) in PBS containing 0.3% Triton X-100 and 5% NGS at 4°C. After several washes (15 min, 3×) in PBS, sections were incubated in the secondary antibodies diluted in PBS containing 0.3% Triton X-100 for 30 min at 37°C (Cy3-conjugated antibody: 1:500, Alexa-conjugated antibody: 1:600).

Sections were cover-slipped with Vectashield (Vector Labs, Burlingame, USA) and stored at –20°C. Slides were observed on a Leica DMRD (Leica Microsystems, Wetzlar, Germany) fluorescence microscope equipped with filter sets that were designed for fluorescein isothiocyanate and Cy3. Sections from double-label experiments were observed on an inverted Zeiss Axiovert 100 M microscope equipped with the LSM 510 META laser scanning confocal module (Carl Zeiss Jena, Jena, Germany).

Two types of controls were performed. To control for unspecific labeling attributable to secondary antibodies, experiments were performed by omitting the primary antibody. This eliminated all labeling. To control for unspecific labeling attributable to the primary antibodies, preadsorption experiments were performed by mixing, in a 20-fold molar excess, a synthetic peptide having the amino acid sequence against which each mGluR antibody had been raised (see below) with the corresponding primary antibody overnight at 4°C. Incubation proceeded as previously described, with the same concentrations for the primary antisera. Labeling was also completely eliminated in these preadsorption controls.

Electron microscopy

The same fixation and cryoprotection procedures were used as described for light microscopy. The retinas were peeled off the filter and embedded in Tissue Tek (Sakura Finetek Europe, Zouterwoude, The Netherlands) in an aluminum boat. The boat was frozen in liquid nitrogen. Transverse frozen sections (30–40 µm thick) were obtained on a freezing microtome and collected in PB at room temperature (equivalent to freeze-thawing).

The retinal sections were incubated for 72 h with diluted antisera (for dilutions, see Table 1). After being rinsed, the

Table 1 Primary antibodies (*LM* light microscopy, *EM* electron microscopy, *aas* amino acids, *PKC* protein kinase C, *mGluR* metabotropic glutamate receptor)

Antibody	Source	Catalog number	Lot	Type	Host	Purification	Antigen	Dilution for LM	Dilution for EM	Blot (kDa) ^a
PKC ^b	Sigma-Aldrich, St. Louis, USA	P5704	0641H4847	Monoclonal	Mouse	Protein A	Bovine, within aas 296–317 of brain PKC	1:500	1:400	80
mGluR1 α	Chemicon, Temecula, USA	AB1551	196011	Polyclonal	Rabbit	Affinity	Rat, C-terminal, aas 1180–1199 (PNVTYASVILRDYKQSSSTL)	1:100	1:100	140
mGluR2/3	Chemicon	AB1553	196011	Polyclonal	Rabbit	Affinity	Rat, C-terminal, aas 860–872 (NGREVVDSTSSL)	1:100	1:100	100 and 190
mGluR5	Chemicon	AB1596	24010139	Polyclonal	Rabbit	Affinity	Rat, C-terminal, aas 1159–1171 (LIIRDYTSQSSSL)	1:100	1:100	116
mGluR7	Upstate, Lake Placid, USA	07–239	25162	Polyclonal	Rabbit	Protein A	Human, C-terminal, aas 899–912 (NSPAAKKKYVSYNN)	1:500	1:500	97

^a Western blots performed by external groups; relative mass values as reported in manufacturer's data sheets^b Produced after Parker et al. 1984 and Young et al. 1988

tissue was incubated in a poly-horseradish-peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG (PowerVision, ImmunoVision Technologies, Daly City, USA). To visualize the peroxidase, the material was incubated in a TRIS-HCl diaminobenzidine (DAB, 0.05%) solution containing 0.03% H₂O₂. The DAB reaction product was subsequently intensified by the gold-substituted silver peroxidase method (Gorcs et al. 1986).

Sections were rinsed in sodium cacodylate buffer 0.1 M (pH 7.4) and post-fixed for 20 min in 1% OsO₄ supplemented with 1.5 % potassium ferricyanide in sodium cacodylate buffer 0.1 M (pH 7.4). After being rinsed in sodium cacodylate buffer, the material was dehydrated and embedded in epoxy resin. Ultrathin sections were observed in a Philips EM201 electron microscope (Philips, Eindhoven, The Netherlands) and/or Technai (CM) 12 electron microscope (FEI Company, Eindhoven, The Netherlands). Sections examined with the Philips electron microscope were counter-stained with uranyl acetate and lead citrate.

Sampling and production of photomicrographs

At least 20 retinal sections obtained from a minimum of five animals were used for a given light microscopy or electron-microscopy experiment (i.e., single-immunolabeling at the light-microscopy level for mGluR1 α). About five photomicrographs were taken from the most representative sections in each experiment.

Light micrographs of the single-label experiments were acquired as TIFF files directly from the Leica microscope by using a Leica 350 F digital B/W camera (1284×1028 pixels, 72 ppi). Light micrographs of the double-label experiments were acquired as TIFF files from the Zeiss LSM 510 META (1048×1024 pixels, 72 ppi).

For electron-microscopy experiments, the same number of retinal sections/animals as described above was examined at the light-microscopical level; labeled sections were chosen for ultra-thin sectioning. A minimum of 40 photoreceptor terminals were observed per ultra-thin section. Micrographs ($n=10-30$) were taken from these sections, depending on the presence and amount of labeling.

Electron micrographs obtained with the FEI electron microscope were directly acquired as TIFF files (1024×1024 pixels, 72 ppi). Electron micrographs obtained from the Philips electron microscope were first printed from the negatives for analysis. The negatives of the prints that were selected for publication were scanned on a sprint scan 4000 scanner (Polaroid Nederland, Breda, The Netherlands) and acquired as TIFF files at 600 ppi.

All TIFF files were resampled at 400 ppi and subsequently re-sized and optimized for brightness and contrast by using Photoshop (Adobe Systems, San Jose, USA).

Immunochemicals

The primary antibodies used in this study were purchased from Chemicon International (Temecula, USA), Sigma-Aldrich (St. Louis, USA), and Upstate (Lake Placid, USA). Details of these antibodies are listed in Table 1. Goat anti-mouse Alexa was obtained from Molecular Probes (Breda, The Netherlands) and goat anti-rabbit Cy3 from Jackson ImmunoResearch Lab (West Grove, USA). For preadsorption controls, custom-made peptides were obtained from Eurogentec (Seraing, Belgium).

Western blotting

Goldfish retinas and brains were homogenized with a Teflon pestle in ice-cold PBS (pH 7.4) containing a tablet of protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) per 50 ml PBS. After homogenization, a TRIS-buffered sample (pH 6.8) solution was added, with final concentrations of: 2% SDS, 5% β -mercaptoethanol, 10% glycerol, 0.005% bromophenol blue. The samples were boiled for 2 min and centrifuged for 10 min at 14,600 g; supernatants were stored at -20°C until use. The diluted samples were boiled for 2 min ($3\times$), cooled, and then spun in a microfuge at 14,600g.

For sodium dodecyl-sulfate polyacrylamide gel electrophoresis, samples were run through a polyacrylamide stacking gel at 20 mA and through a 13% polyacrylamide gel at 30 mA. Protein standards (Bio-Rad Laboratories, Veenendaal, The Netherlands) were run in adjacent lanes. Gels were electroblotted on a poly-vinylidene-di-fluoride blot membrane (Millipore) overnight at a constant current of 80 mA. After being rinsed with a 0.5 M TRIS buffer containing 1.5 M NaCl and 5% Tween 200, the membrane was blocked in the same buffer containing 2% dry milk for 1 h.

Subsequently, the membrane was incubated for 1 h in the primary antibodies (1:100), washed in the TRIS buffer, incubated in an HRP-conjugated goat anti-rabbit IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, USA), and washed in PBS. The immunoreaction was visualized by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, USA) by using Kodak film. Exposures times were between 1 and 5 min.

Results

In this study, we aimed at a qualitative description of the mGluR composition in the goldfish OPL. To have a parameter for comparisons with regard to ON BC labeling with the antibodies against the various mGluRs, we first investigated the immunoreactivity pattern yielded by the PKC antibody, a widely used ON BC marker. Therefore, the labeling for PKC will be described first, followed by each of the mGluRs studied.

Immunolabeling for PKC

The PKC antibody was characterized by Western blot previously in a number of studies (Negishi et al. 1988; Suzuki and Kaneko 1990; Yazulla and Studholme 1992) and yielded similar results in various species (Table 1).

Light microscopy Immunolabeling for PKC in the goldfish retina at the light-microscopical level has been described in detail elsewhere (Suzuki and Kaneko 1990). Briefly, two populations of ON BCs were labeled: cells with small somata in the middle of the inner nuclear layer (INL) and small axonal terminations (cone-driven ON BCs), and cells with large flask-shaped somata and bulbous axon terminals (ON MBCs).

Electron microscopy: rod synapses In the rod synaptic complex, the antibody against PKC labeled invaginating dendrites at the central position and processes at some distance from the ribbon (Fig. 1a–c, arrowheads). The labeled structures were elongated (Fig. 1a), u-shaped (Fig. 1b), or round (Fig. 1c) and were found either directly opposite to the synaptic ribbon (Fig. 1a,b) or a little distance away from it (Fig. 1c). Dendrites with these characteristics and at these locations in the cyprinid retina were previously shown to belong to ON MBCs (Scholes 1975; Stell 1967, 1976, 1978; Saito et al. 1983, 1985; Klooster et al. 2001; Yazulla and Studholme 2001).

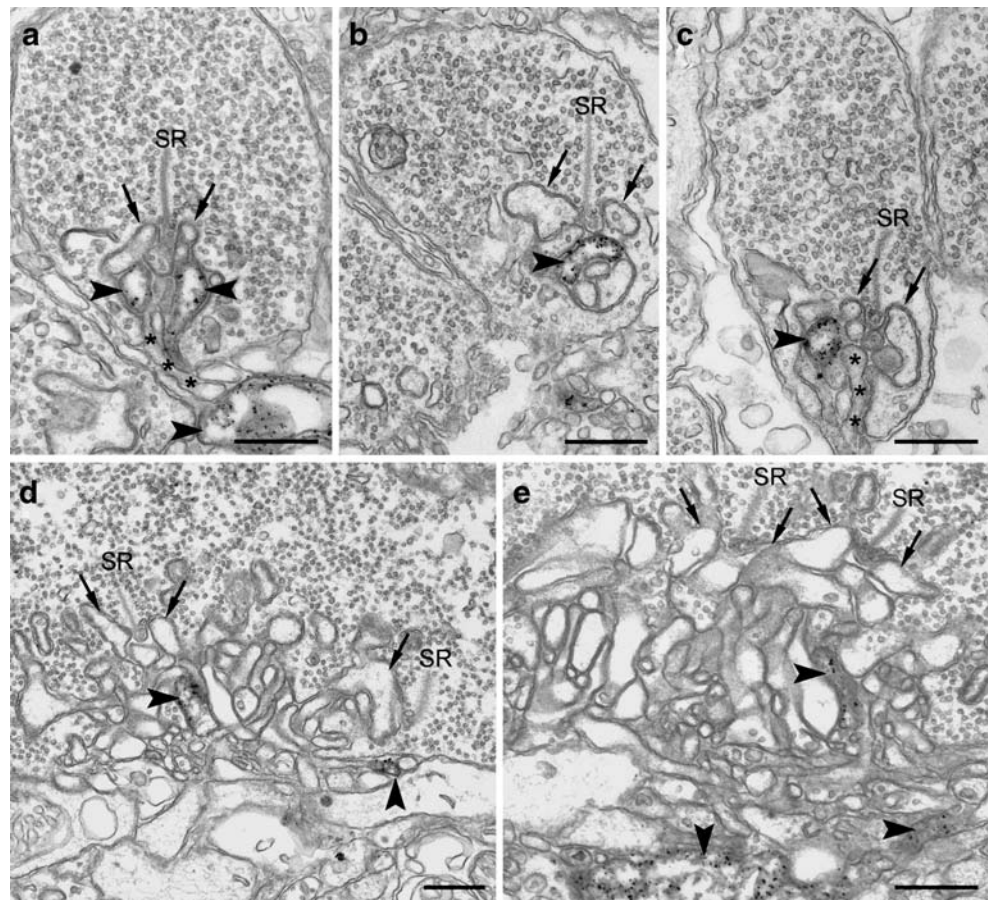
Lateral processes immediately adjacent to the pre-synaptic ridge, previously classified as dendrites of rod-driven HCs (Stell 1967, 1976; Scholes 1975) were not labeled (Fig. 1a–c, arrows). Some invaginating processes were also not labeled (Fig. 1a,c, asterisks); their position and fine structure, in combination with the lack of labeling, suggested that they were either dendrites of OFF-type MBCs or rod-driven HCs (Stell 1967, 1976; Saito et al. 1983, 1985).

Electron microscopy: cone synapses PKC-immunoreactive structures were not abundant in the vicinity of the cone pedicles. When present, the labeled structures were never associated with the synaptic ribbon but were instead localized at the base of the cone pedicle (Fig. 1d,e, arrowheads). These were probably ON MBC processes approaching the cone pedicle. In the closely related carp, dendrites of horseradish peroxidase-injected ON MBCs have been found at similar sites in the cone synaptic complex (Saito et al. 1983, 1985). Lateral elements were unlabeled (Fig. 1d,e, arrows).

Immunolabeling for mGluR1 α

Western blotting The mGluR1 α antibody did not recognize any proteins in the goldfish retina, possibly because of the low concentration of the receptor in this tissue. The antibody

Fig. 1 Ultrastructural localization of protein kinase C (PKC) in the outer plexiform layer. In the rod spherules (**a–c**), invaginating dendrites of putative mixed-input ON-type bipolar cells (ON MBCs) were positively labeled (*arrowheads*). Lateral elements of horizontal cells (HCs) were unlabeled (*arrows*), as were some invaginating processes that possibly belonged to OFF-type MBCs (*asterisks*). In the cone pedicles (**d, e**), PKC immunoreactivity was observed in small invaginating profiles at the base of the pedicles (*arrowheads*). These structures were never associated with the synaptic ribbons (*SR*). HC lateral elements were unlabeled (*arrows*). Bars 0.5 μ m



produced a diffuse band at approximately 150 kDa in goldfish brain and rat samples (Fig. 2c), close to the predicted molecular mass of mGluR1 α (Tables 1, 2). The immunoreactive protein found in the rat retina was faint, probably reflecting the restricted expression of this receptor in retinal tissue.

Light microscopy mGluR1 α immunoreactivity in the goldfish retina was concentrated in the OPL (Fig. 2a) and outer half of the INL in which the somata of some bipolar cells were labeled (Fig. 2a, arrowheads). A confocal examination of the immunofluorescence pattern yielded by this antibody revealed diffuse labeling in the INL, inner plexiform layer (IPL), and ganglion cell layer (GCL; Fig. 2d). Red and green images of a confocal view were taken from a retinal slice double-labeled for mGluR1 α (Fig. 2d) and PKC (Fig. 2e); the yellow areas in the merged image (Fig. 2f) indicated the strong colocalization in ON BC processes in the OPL. However, mGluR1 α immunoreactivity in this layer was not restricted to PKC-positive processes. Furthermore, the small PKC-positive ON BCs were not positive for mGluR1 α (Fig. 2d,e, asterisks). Conversely, the mGluR1 α -antibody labeled structures (presumably amacrine cell somata) in the inner border of the INL that were not PKC-positive (Fig. 2d,e, circles).

Electron microscopy: rod synapses In the rod spherule, mGluR1 α immunoreactivity was present at invaginating dendrites in close apposition to the synaptic ribbons (Fig. 3a–d, arrowheads). These processes were elongated (Fig. 3a), ellipsoidal (Fig. 3b), goblet-shaped (Fig. 3c), or round (Fig. 3d), consistent with ON MBC dendrites. Roughly two invaginating dendrites were stained per rod spherule; the remaining invaginating dendrites (Fig. 3a,b, asterisks) were not labeled. These were possibly OFF MBCs, rod-driven HCs, or a subtype of ON MBC negative for mGluR1 α .

The antibody also labeled single lateral elements facing the synaptic ribbon (Fig. 3d, thick arrow). Whenever a lateral element was labeled, the other one was unlabeled (Fig. 3d, arrow). Although processes in the lateral position are classically attributed as belonging to HCs (Stell 1967, 1976), we could not fully exclude the possibility that some of the labeled processes were aberrant ON MBC dendrites.

Electron microscopy: cone synapses In the cone pedicles, various types of post-synaptic structures were labeled. In addition to small invaginating processes (Fig. 3e,f, arrowheads), which could belong to BCs, mGluR1 α immunoreactivity was also present at one lateral dendrite of some cone triads (Fig. 3e, thick arrows). The position and fine structure of these dendrites were consistent with those of cone-driven

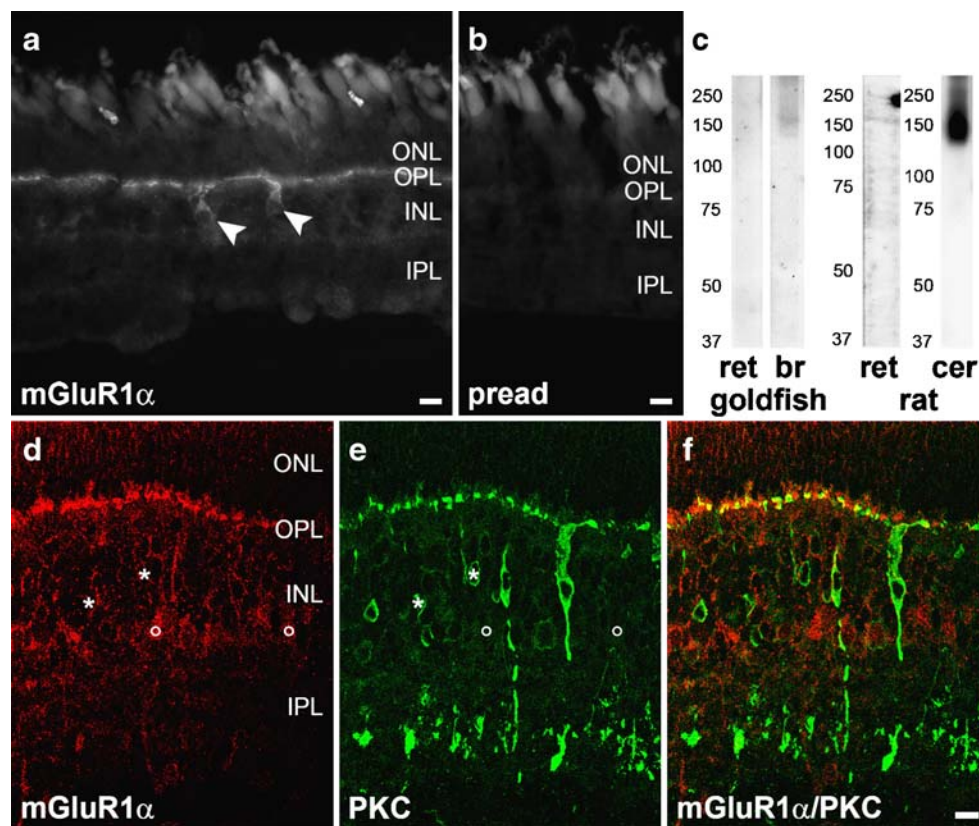


Fig. 2 Immunolabeling for mGluR1 α (ONL outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer). **a** Immunoreactivity was concentrated at the OPL and outer half of the INL in which some BC somata were partially labeled (arrowheads). **b** Preadsorption with the control peptide (pread) abolished all labeling. **c** Western blot of total protein from goldfish retina (ret), goldfish brain (br), rat retina (ret), and rat cerebrum (cer). A protein at around 150 kDa, close to the predicted molecular mass of mGluR1 α , was detected in goldfish brain, rat retina, and rat cerebrum. **d**, **e** Red and

green channel confocal images of the same tissue section double-labeled with antibodies against mGluR1 α (Cy3, **d**) and PKC (Alexa, **e**). Some structures were positive for mGluR1 α but not for PKC (circles). Some small BCs were positive for the ON BC marker but not mGluR1 α (asterisks). **f** Merged image of the double-labeling experiment. A band of mGluR1 α immunoreactivity in the OPL partially overlapped with the dendritic processes of PKC-positive cells. Areas of overlap between the two proteins appear yellow. Bars 10 μ m

HCs (Stell 1967, 1976; Sakai and Naka 1983). When one lateral HC dendrite in a triad was positive for mGluR1 α , the opposite HC dendrite was not (Fig. 3e, thick and thin arrows, respectively). A few invaginations in the cone pedicle containing discontinuous patches of electron-dense material in the membrane and resembling HC spinules (Wagner 1980) exhibited mGluR1 α immunoreactivity (Fig. 3f, thick arrows).

Immunolabeling for mGluR2/3

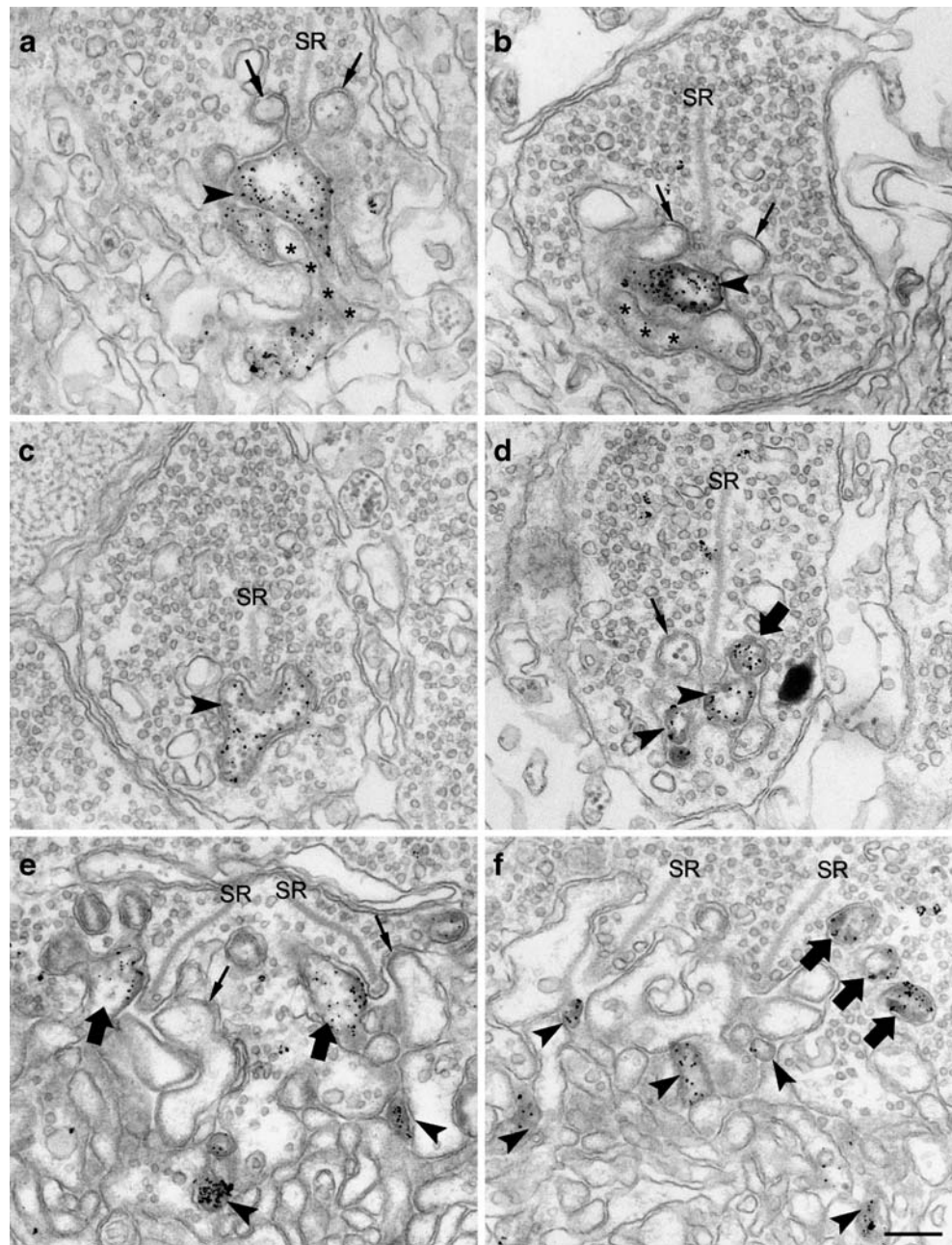
Western blotting The antibody against mGluR2/3 reacted with proteins of approximately 100 kDa in goldfish brain, rat retina, and rat cerebrum (Fig. 4c). This is the predicted molecular mass of mGluR2 and mGluR3 (Tables 1, 2). In the goldfish retina, only a protein at about 200 kDa was detected, which also appeared in the other three tissue samples. This probably represents receptor dimers (Poli et al. 1999), which occur naturally in many G-protein-coupled receptors,

including mGluRs (Bouvier 2001; Kubo and Tateyama 2005; Pin et al. 2005; Poli et al. 2003; Beraudi et al. 2007). In goldfish and rat retinas, a smaller band at around 40 kDa was detected.

Light microscopy Immunocytochemistry with the mGluR2/3 antibody revealed signal in the OPL and INL and strong labeling in the IPL. Tiny discontinuous immunoreactive bands were visible in the OPL (Fig. 4a, thick arrows). Confocal images showed a similar pattern, with a slightly stronger OPL labeling (Fig. 4d, thick arrows). Clearly labeled somata could not be distinguished, although immunolabeling in the INL was concentrated in patches. Immunoreactivity for PKC (Fig. 4e) did not colocalize with mGluR2/3 (Fig. 4d), as can be seen by the absence of yellow areas in the merged confocal image (Fig. 4f).

Electron microscopy: rod synapses No immunoreactive processes could be identified in the rod synaptic terminals.

Fig. 3 Ultrastructural localization of mGluR1 α in the OPL. In the rod terminals (**a–d**), ON MBC invaginating processes at the summit of the synaptic ribbon (SR) were positively labeled (arrowheads). Some invaginating processes were not labeled (asterisks); these might belong to OFF MBCs, to rod-driven HCs, or to a subtype of ON MBC negative for mGluR1 α . Occasionally, a laterally positioned dendrite showed mGluR1 α immunoreactivity (thick arrow in **d**). In the cone pedicles (**e, f**), mGluR1 α immunoreactivity was found in small invaginating profiles that lay at the base of the pedicles or close to the ribbon (arrowheads) and that might have originated in BCs. In addition, a HC dendrite occupying the lateral position of the triads was positively labeled (thick arrows in **e**), whereas the other lateral element was unlabeled (arrows). The antibody also labeled HC spinules in the cone terminals (thick arrows in **f**). Bar 0.25 μ m



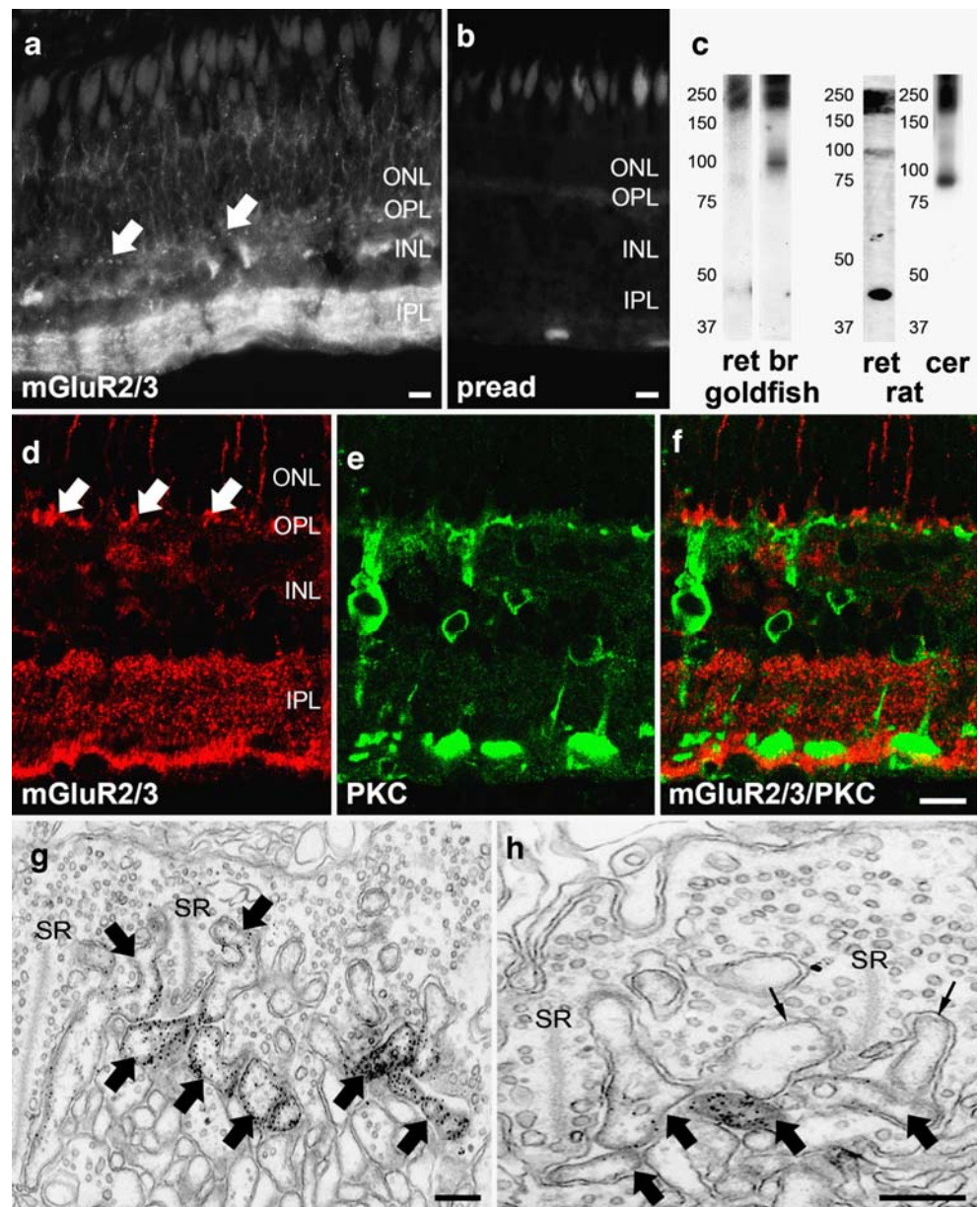
Electron microscopy: cone synapses HC dendrites occupying both the lateral and central position were positive for mGluR2/3 in only a few cone pedicles (Fig. 4g–h, thick arrows). The fine structure of the central processes was consistent with that of HC dendrites because of their large dimensions compared with BC dendrites, cytoplasm devoid of organelles, some electron-dense amorphous material applied to the cytoplasmic surface, and concavity of the surface facing the synaptic ribbon (Stell 1967, 1976; Scholes 1975). No mGluR2/3 immunoreactivity was seen in structures resembling BC dendrites, in agreement with our light-microscopical findings.

Immunolabeling for mGluR5

Western blotting Immunoblots for mGluR5 revealed a protein at around 100 kDa in goldfish retina and brain and in rat cerebrum (Fig. 5c). An additional band at 60 kDa was detected in the goldfish samples and in the rat retina. A heavier protein of around 145 kDa was detected in rat retina and cerebrum, roughly corresponding to the deduced molecular mass of rat mGluR5 (Koulen et al. 1997).

Light microscopy The mGluR5 antibody labeled vertical bands at the outer nuclear layer (ONL) in both conventional

Fig. 4 Immunolabeling for mGluR2/3 (ONL outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer). **a** Immunoreactivity was seen as tiny bands in the OPL (*thick arrows*), irregular patches in the INL, and diffuse labeling in the IPL. **b** Preadsorption with the control peptide (*pread*) abolished all labeling. **c** Western blot of total protein from goldfish retina (*ret*), goldfish brain (*br*), rat retina (*ret*), and rat cerebrum (*cer*). A protein at around 100 kDa, close to the predicted molecular mass of mGluR2 and mGluR3, was detected in goldfish brain, rat retina, and rat cerebrum. The antibody also detected a protein at 200 kDa (probably receptor dimers) in all four tissues. **d, e** Red and green channel confocal images of the same tissue section double-labeled with antibodies against mGluR2/3 (Cy3, **d**) and PKC (Alexa, **e**). **f** Merged image of the double-labeling experiment. No colocalization with ON BCs was found. Bars 10 μ m. **g, h** In a few cone pedicles, mGluR2/3 immunoreactivity was found in HC central and lateral elements of the cone triad (*thick arrows*). Note the unlabeled lateral processes (*thin arrows*) and synaptic ribbons (SR). Bars 0.5 μ m



(Fig. 5a, open arrows) and confocal microscopy (Fig. 5d, open arrows) and yielded granular labeling in the OPL, INL, and IPL. Some HC somata were positively labeled at the outer margin of the INL (Fig. 5a,d, thick arrows). There was no colocalization with PKC (Fig. 5d–f).

Electron microscopy Ultrastructurally, mGluR5 immunoreactivity was seen in longitudinally oriented structures between photoreceptor terminals (Fig. 5g, open arrows). This pattern was consistent with previous reports concerning the localization and ultrastructural appearance of Müller cell processes (Villegas 1960; Lasansky 1961; Pedler 1963; Miller and Dowling 1970). Furthermore, it resembled closely that of immunolabeling for glial fibrillary acidic protein (Fig. 5h, open arrows), which is exclusively

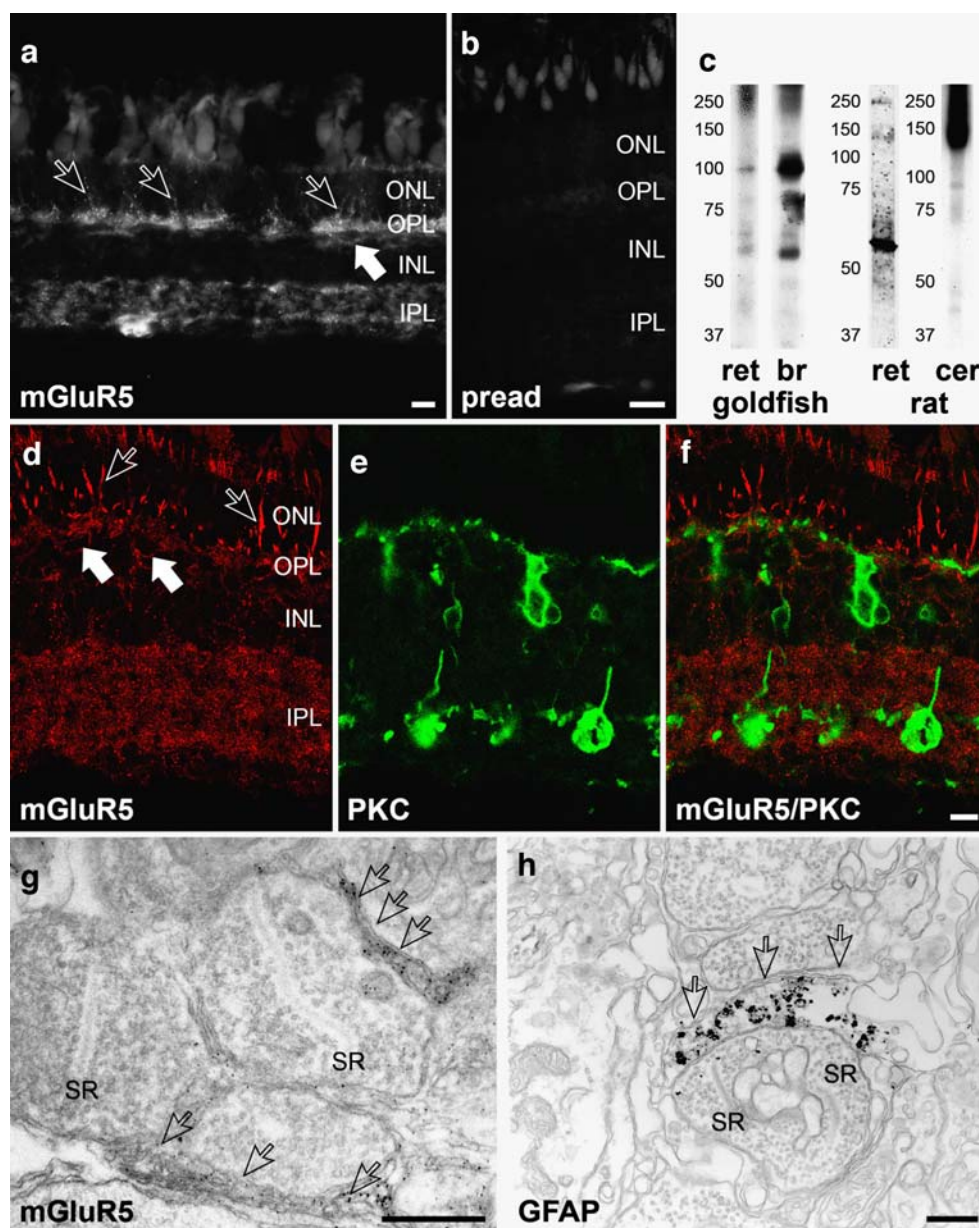
expressed by glial cells (Bignami and Dahl 1977; Liepe et al. 1994).

Immunolabeling for mGluR7

Western blotting A protein of approximately 50 kDa was labeled in goldfish and rat samples, with an additional band of around 75 kDa in the goldfish brain. In the rat retina, a protein of 200 kDa was also recognized. A clear band could not be distinguished in rat cerebrum, although there was a larger precipitation around 50 kDa (Fig. 6c), at some distance from the value reported by the producer (Table 1).

Light microscopy The antibody against mGluR7 immunoreactivity yielded strong labeling in the OPL (Fig. 6a,

Fig. 5 Immunolabeling for mGluR5 (*ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer). **a** Immunoreactivity was concentrated in the ONL (*open arrows*), with some granular labeling in the OPL, INL, and IPL (*thick arrow* labeled HC body). **b** Preadsorption with the control peptide (*pread*) abolished all labeling. **c** Western blot of total protein from goldfish retina (*ret*), goldfish brain (*br*), rat retina (*ret*), and rat cerebrum (*cer*). A protein around 100 kDa was detected in the goldfish tissue and rat brain. The antibody also a smaller band (60 kDa) in the goldfish samples and in the rat retina, and a heavier band (145 kDa) in the rat retina and cerebrum. **d, e** Red and green channel confocal images of the same tissue section double-labeled with antibodies against mGluR5 (Cy3, **d**) and PKC (Alexa, **e**). **f** Merged image of the double-labeling experiment, showing no colocalization. Bars 10 μ m. **g** Labeling is restricted to processes ensheathing photoreceptor terminals in the ONL (*open arrows*). **h** A similar labeling pattern (*open arrows*) is obtained with the monoclonal antibody against glial fibrillary acidic protein (*GFAP*; clone G-A-5, Sigma-Aldrich; 1:400), which labels exclusively Müller cells (*SR* synaptic ribbon). Bars 0.5 μ m



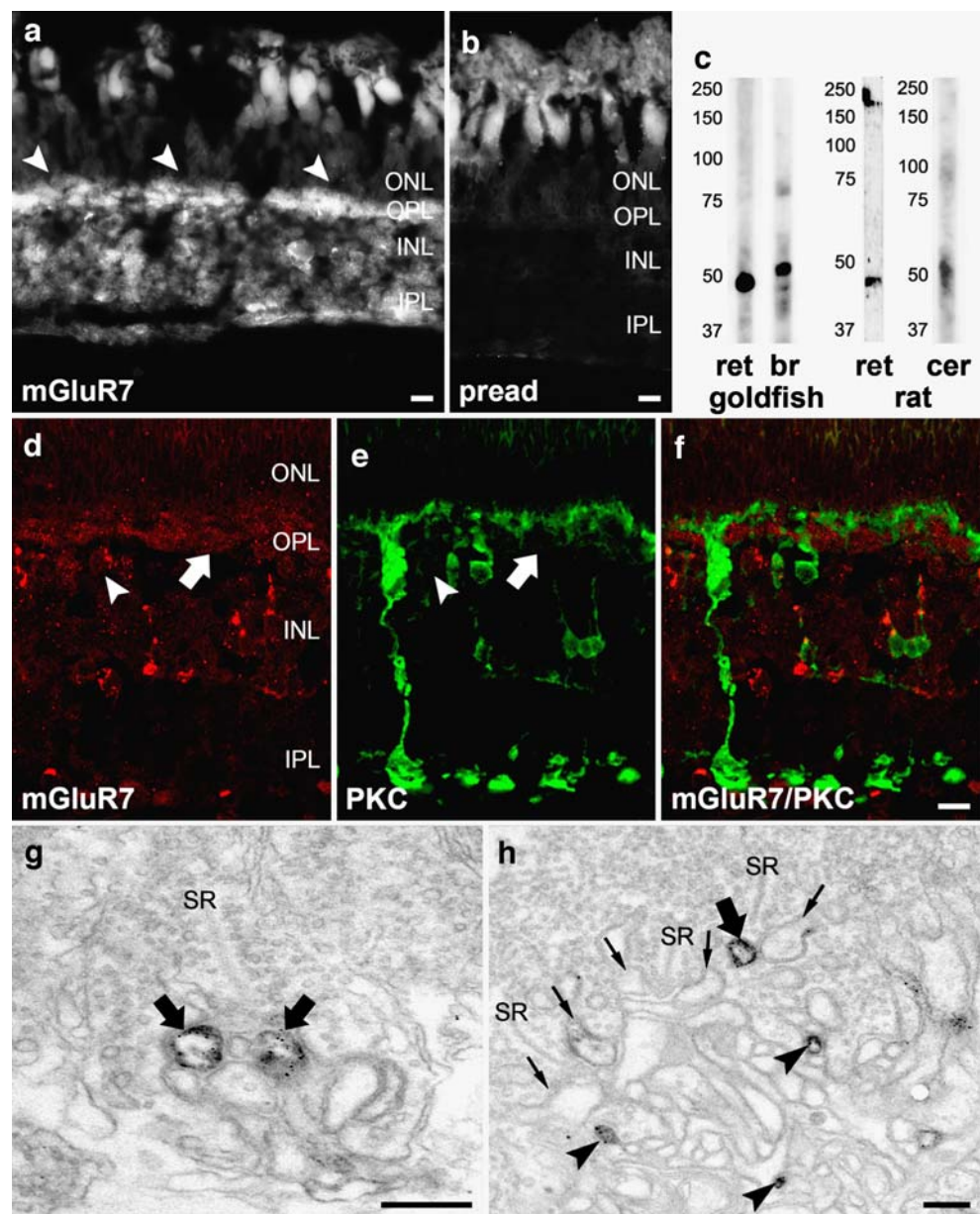
arrowheads) and granular labeling at the INL, IPL, and GCL. Occasionally, positive amacrine and ganglion cell somata were seen. Confocal images of double-labeling with PKC (Fig. 6d–f) did not show colocalization: mGluR7-positive areas were concentrated close to the outer border of the INL, immediately below the band of PKC-positive ON BC dendrites. Some mGluR7-positive somata that were not labeled for PKC could be identified in the outer half of the INL; their form and position was consistent with that of HCs (Fig. 6d,e, thick arrow) and OFF BCs (Fig. 6d,e, arrowhead).

Electron microscopy: rod synapses Lateral elements of the rod triads were labeled, consistent with the labeling of

putative HCs reported above (Fig. 6g, thick arrows). We found no evidence of MBC labeling in rod spherules.

Electron microscopy: cone synapses A few small processes away from the ribbons were labeled by the mGluR7 antibody (Fig. 6h, arrowheads). Their small profiles resembled those of BCs. This observation, added to the finding that the antibody labeled PKC-negative BC somata (Fig. 6d,e, arrow), suggested that these were OFF BC profiles. Lateral elements (Fig. 6h, thick arrow) and spinules were also labeled, consistent with HC labeling at the light-microscopical level (Fig. 6d, e, thick arrows). These labeled HC processes were however rare; most of the lateral elements were not positive (Fig. 6h, thin arrows).

Fig. 6 Immunolabeling for mGluR7 (ONL outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer). **a** Immunoreactivity was concentrated in the OPL (arrowheads), with some granular labeling in the INL, IPL, and GCL. **b** Preadsorption with the control peptide (*pread*) abolished all labeling. **c** Western blot of total protein from goldfish retina (*ret*), goldfish brain (*br*), rat retina (*ret*), and rat cerebrum (*cer*). A pronounced band at around 50 kDa was produced in all four samples. Additional immunoreactive proteins were found at 75 kDa in the goldfish tissue and at 200 kDa in the rat retina. **d, e** Red and green channel confocal images of the same tissue section double-labeled with antibodies against mGluR7 (Cy3, **d**) and PKC (Alexa, **e**). A BC soma (arrowheads) and an HC soma (thick arrows) were positive for mGluR7 but negative for PKC. **f** Merged image of the double-labeling experiment, showing no colocalization in ON BCs. **g** In rod spherules, lateral elements were labeled (thick arrows). **h** In the cone pedicles, small profiles not related to the synaptic ribbons (SR) were positive (arrowheads), as were lateral elements of the triads (thick arrow). Note the unlabeled lateral elements (thin arrows). Bars 0.25 μ m



Discussion

Summarizing scheme

This study shows that mGluRs of all three groups can be found in the goldfish OPL. The main findings are summarized in Fig. 7. Cell types have been identified based on the results obtained at both light-microscopy and electron-microscopy levels.

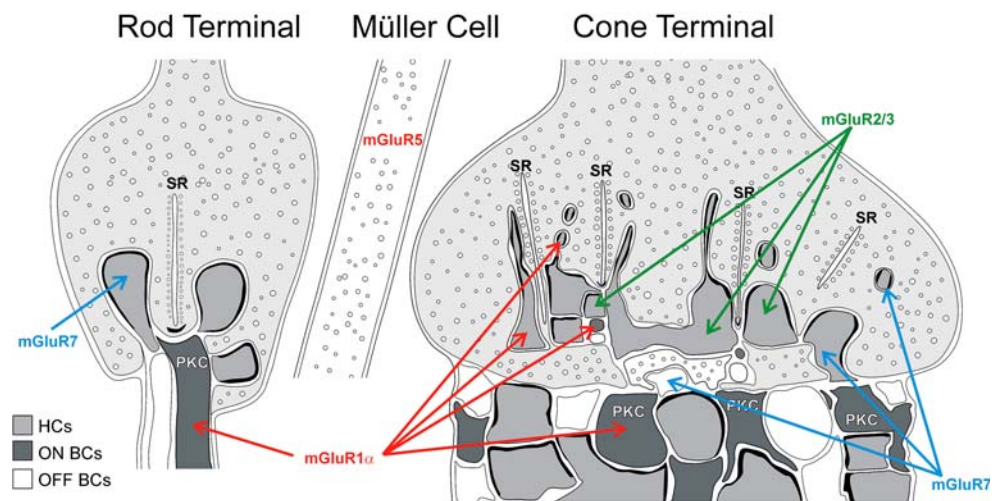
In the rod spherule, PKC labels invaginating dendrites of putative ON MBCs. The mGluR1 α antibody labels similar structures and some lateral elements. Lateral elements of rod triads are also positive for mGluR7. Müller cell processes between the rod spherules are positive for mGluR5. We have found no evidence for mGluR2/3 in the rod synaptic complex.

In the cone pedicles, PKC labels fine dendrites at the base of the terminal or invaginating structures away from the ribbons. Elements at similar positions and with similar fine structure are positive for mGluR1 α and mGluR7. BC central processes in the cone triads are positive for mGluR1 α . Spinules are positive for mGluR1 α and mGluR7, and lateral elements are labeled by the mGluR1 α , mGluR2/3, and mGluR7 antibodies. Finally, HC central elements are positive for mGluR2/3.

Specificity of the antibodies

No data are available on the amino acid and/or nucleotide sequences of goldfish mGluRs. The closest available information is from the zebrafish. This database is however not fully completed, and the various zebrafish mGluRs have not been

Fig. 7 Representation of a rod spherule, a Müller cell process, and a cone pedicle, depicting the localization of PKC and the patterns yielded by the various mGluR antibodies (*red* group I mGluRs, *green* group II mGluRs, *blue* group III mGluRs, *SR* synaptic ribbon)



unambiguously identified (Table 2). The finding of candidates for zebrafish mGluR2 and mGluR6 has been reported, but the actual nucleotide and amino acid sequences have not been disclosed (Bjarnadottir et al. 2005).

We therefore used multiple data sources to infer the specificity of the antibodies used in this study: Western blots and BLAST searches in the zebrafish genome for the amino acid sequences in Table 1 (at <http://www.ncbi.nlm.nih.gov/BLAST/>), the sequence alignments (with http://www.ch.embnet.org/software/LALIGN_form.html and <http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>), and the molecular mass calculations (at <http://www.basic.northwestern.edu/biotools/proteincalc.html>). We also “BLASTed” the proteins retrieved from the zebrafish database against all other species available, in order to check for protein similarities among species.

To choose good candidates for fish mGluRs among the BLAST hits, we relied on the scores and E-values; the cutoff was $E < 0.01$. We adopted a stringent significance level, because the chance of obtaining random matches with short amino acid sequences was relatively large. All scores used for these comparisons were higher than 1,000. For the C-terminal mGluR5 peptide, the cutoff was lowered to $E = 0.35$, since no candidates could be found with the same stringency as for the remainder of the peptide sequences.

As summarized in Table 2, all of the anti-mGluR antibodies used in this study were raised against polypeptides that yielded mGluR-like hits when “BLASTed” against the zebrafish database. Two homologs were found for mGluR1, but one of these (XP_001346460) was an incomplete sequence that might have corresponded to a fragment of the other (XP_690446), since they only differed by one amino acid and their respective coding sequences by two nucleotides. For mGluR2/3, three putative mGluRs were identified (Table 2), but one of them had a slightly smaller molecular mass than the other two (85 kDa). We did not however

detect multiple proteins around 80–100 kDa in our immunoblots, which suggested that XP_001341692 was not present in the goldfish retina.

According to the database search, the C-terminal peptides of mGluR5 and mGluR7 have one fish homolog each. The mGluR5 candidate shows lowest amino acid sequence similarity at the C-terminus, but the lack of overlapping expression patterns between mGluR1 and mGluR5 in the OPL of the goldfish retina excludes cross-reactivity of anti-mGluR5 and mGluR1.

In summary, we feel confident about the specificity of the commercially available anti-mGluR peptide antibodies, since the carboxytermini of mammalian and fish mGluR homologs are nearly identical, and the molecular masses of fish and mammalian homologs are similar (Table 2).

mGluRs of Müller cells

The finding of mGluR5 in Müller cells is in agreement with two previous studies of isolated Müller cells from the salamander; these studies have shown that activation of group I mGluRs has two major effects. First, it leads to the closure of the K^+ conductance of these cells, which is by far the largest in Müller cells (Schwartz 1993). Since their glutamate uptake is both voltage-dependent (Brew and Attwell 1987; Schwartz and Tachibana 1990) and regulated by the internal K^+ concentration (Barbour et al. 1988), one can imagine that mGluR5, by modulating this K^+ conductance, indirectly modulates glutamate uptake by changing the membrane potential and/or internal K^+ concentration.

The second effect of group I mGluR activation in Müller cells is the generation of Ca^{2+} waves by triggering the release of Ca^{2+} from internal stores (Keirstead and Miller 1997). The physiological consequences of this effect are not completely clear, but they could contribute to lateral signaling among Müller cells, as in astrocytes (Kim et al. 1994).

mGluRs of HCs

Here, we show that rod and cone-driven HCs of the goldfish express mGluRs from all three groups, although the exact mGluR composition of these cells differs slightly from reports in other species (i.e., no mGluR1 in cat HCs: Cai and Pourcho 1999; no mGluR2/3 in catfish HCs: Gafka et al. 1999).

We have found that lateral elements in the rod triads are positively labeled for mGluR1 α and mGluR7. Since the labeling for mGluR1 α is rare, we cannot at this point positively identify these processes as belonging to either rod-driven HCs or MBCs. In the cone pedicle, mGluRs seem to be expressed in a highly organized manner. mGluR1 α and mGluR7 tend to be localized to spinules and to dendrites occupying the lateral position in the cone triad, whereas mGluR2/3 has been found at both lateral and central processes directly opposing the synaptic ribbon.

This extremely ordered pattern indicates that either a single cone-driven HC type expresses distinct mGluRs in different dendrites, or that the expression of a given mGluR is restricted to a certain subpopulation of HCs (i.e., the distribution is cell-type-specific). In cyprinid retinae, three physiological and morphological HC types have been described (MacNichol and Svætichin 1958; Norton et al. 1968; Stell et al. 1982); one could easily imagine that each HC class expresses only one or two mGluRs at their dendritic tips.

The light responses of HCs are mediated by AMPA (2-amino-4-phosphonobutyric acid)/kainic acid receptors (Lasater and Dowling 1982; Slaughter and Miller 1983; Zhou et al. 1993) and are not abolished by mGluR agonists or antagonists (Yang and Wu 1989; Luo and Liang 2003), showing that mGluRs are not implicated in the direct generation of light responses. They are most likely involved in the modulation of the light-driven conductance of these cells. In the fish retina, for instance, mGluR agonists have been shown to modify both light-modulated (Nawy et al. 1989; Yasui et al. 1990; Takahashi and Copenhagen 1992) and voltage-gated conductances of cone-driven HCs (Dixon and Copenhagen 1997; Linn and Gafka 1999; Gafka et al. 1999).

mGluRs of ON MBCs

The labeling of ON MBC processes, in the rod spherule, with the antibody against mGluR1 α corroborates previous studies in fish (Klooster et al. 2001; Yazulla and Studholme 2001; Yazulla et al. 2001) and mammalian (Koulen et al. 1997) species, although it differs from the situation in the cat retina, in which mGluR1 α is pre-synaptic to rod-driven BCs (Cai and Pourcho 1999). Since no evidence has as yet been presented for the participation of group I mGluRs in the generation of the light responses of ON BCs, one can only speculate that this protein plays a modulatory role in the first

synapse. In many parts of the central nervous system, group I mGluRs are involved in controlling the intracellular calcium concentration and membrane channel activity (for a review, see Fagni et al. 2000). In the spinal cord neurons of the lamprey, for instance, mGluR1 has been shown to regulate the resting membrane potential and thus the excitability of the cells by controlling a leak current (Kettunen et al. 2003).

Although there is physiological evidence for the existence of a group III mGluR in fish ON MBCs (Shiells et al. 1981; Nawy and Copenhagen 1987; Kamermans et al. 2004), we have not been successful in obtaining specific immunoreactivity with the antibodies commercially available against (mammalian) mGluR4, mGluR6, and mGluR8. Furthermore, there is as yet no candidate for mGluR6 in the zebrafish database, which makes the production of custom-made antibodies against this protein difficult. One would however expect these cells to express an mGluR6-like receptor, given the many physiological similarities between the rod-driven conductance in fish and mammalian ON BCs.

Whether and in what manner this mGluR6-like receptor and the mGluR1 α -like protein interact in ON MBCs is still to be resolved. G-protein-coupled receptors can interact at many levels. For instance, gamma-aminobutyric acid_B receptors form complexes with mGluR1 α dimers and thereby regulate the sensitivity of the latter to glutamate (for a review, see Kubo and Tateyama 2005). Interactions between mGluRs, however, have so far not been described. Another level of interaction lies within the second-messenger cascade. Until recently, only one G-protein (G_o α) had been described in ON BCs (Vardi et al. 1993), which suggests that mGluR1 α and mGluR6 share (part of) the same intracellular machinery. Nonetheless, a second G-protein (G_{i2} α) has been shown to coexist with G_o α in rat ON BCs and not to couple to mGluR6 (Tian and Kammermeier 2006), providing an independent G-protein signaling pathway within these cells; this pathway might involve mGluR1 α .

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